the case in pharmacological work.

The apparatus was designed to be simple and reliable. The light source was chosen to be a powerful light emitting diode, emitting in the red. The advantage of using red light is that the interfering effects of other chromatophores than the melanophores, i.e. xantophores and erythrophores, can be virtually eliminated [10].

The recording obtained by the use of the scale photometer (see Figs. 2A and 3A, B) are comparable to the recordings obtained by the use of other methods [e.g. 4, 6]. It can be noted that the change of transmission following maximal stimulation of the melanophores by drugs (Fig. 2A) or by electrical field stimulation (Fig. 3A) is of comparable size. We have previously shown, by the use of tetrodotoxin [4], that the electrical field stimulation applied in this work activates intrinsic nerves only and not the melanophores directly. The aggregating response to repeated electrical stimulation (Fig. 3B) shows a high degree of reproducibility, which may be of importance in experiments when previously induced responses are used as controls to responses elicited after treatment.

The scale photometer is especially useful when concentration response curves are obtained since several multi sample holders (with five cuvettes each) can be used simultaneously. In Figure 2B the aggregating responses elicited by two adrenergic agonists are shown as a function of concentration; the response to each agonist was evaluated either by the ocular or the photometric method. It can be concluded from the figure that the two methods give very similar results, especially if the deviations are taken into consideration. Furthermore, the form of the curve for the two agonists agree regardless of method. It is evident from the curves in Figure 2B that the deviations are substantial, this appears however to be more a characteristic of the scale melanophores themselves since similar deviations appear regardless of the method applied.

We have developed a method which apply a new photometric apparatus for the assessment of the state of pigment aggregation in melanophores. This apparatus, a scale photometer, allows a methodology that is simple and time-efficient and the method could find potential applications especially in the pharmacological field where it could, e.g., complement existing models for characterization of alpha$_2$-selective drugs. The method could also be of potential value as an assay during purification of pertussis toxin [11] or even used as part of a diagnostic method for whooping cough.

ACKNOWLEDGMENT

The excellent technichal assistance by Mr. P.-E. Sterner is gratefully acknowledged. We also wish to thank Prof. J. O. Strömberg at Kristineberg Marine Biological Station for placing laboratory resources at our disposal. Medetomidine was a gift from Farmos Group Ltd, Turku, Finland. This work was supported by grants from the Swedish Technical Development Board (86–4494), Trion AB and Centrala Försöksdjursnämnden (87–18).

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ZOOLOGICAL SCIENCE 5: 965–971 (1988)

Non-Synaptic Release of Transmitter-Containing Vesicles from the Enteric Neurons of the Rat Small Intestine

Yasuhisa Endo

Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo, Kyoto 606, Japan

ABSTRACT—Exocytotic release of transmitter-containing vesicles from the neurons of the rat small intestine was investigated by electron microscopy using a perfusion method of high K^+ Ringer solution to stimulate release of transmitters. Omega-shaped figures suggestive of exocytosis of large cored vesicles were frequently found at the non-synaptic sites. Exocytosis occurred not only in the surface of nerve fascicles but also in the sites facing other neuronal processes and Schwann cell's processes. These results suggest that the terminal varicosities may not always release their chemical messengers only in the sites directly facing the target tissues.

INTRODUCTION

It is generally accepted that neurons have various chemical messengers as neurotransmitters and release them toward the effector cells through synapses. However, recent electron microscopic studies demonstrated the occurrence of non-synaptic release of neurotransmitters from the neurons in the central nervous system [1] and the adrenal medulla [2].

Most of the enteric neurons of mammals have various neuropeptides which are packed in large cored vesicles (cf. [3]). Several transmission electron microscopic studies on the enteric nervous system have been done, but exocytotic release of large cored vesicles from the neurons has not been described (cf. [4]). Recently, Endo and Kobayashi [5] studied on the three-dimensional structure of the autonomic groundplexus in the small intestine of guinea pigs, by scanning electron microscopy using a HCl-digestion method to eliminate connective tissues. They revealed that the terminal portion of the enteric nervous system was a continuous network of unmyelinated nerve fascicles and the many, varicose neuronal processes were exposed to the surface of unmyelinated nerve fascicles. They postulated that these exposed area of neuronal processes may be the sites of interaction between the enteric neurons and their effector cells.

In the present study, I investigated the exocytotic release of transmitter-containing vesicles from the neurons of the rat small intestine by transmission electron microscopy. In order to stimulate the exocytotic release of them, perfusion of high K^+ Ringer solution was applied (cf. [6]). Tannic acid-glutaraldehyde-osmium tetroxide fixation is known as an useful method to demonstrate the exocytotic figures of secretory granules, because this fixation renders the extracellular secretory substances highly electron dense and does not affect the ultrastructure of other cellular elements [7, 8]. This fixation method was used in this study, combining with a perfusion of high K^+ Ringer solution.

MATERIALS AND METHODS

Adult male Wistar rats (100–200 g body weight) were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Three animals were perfused with 50 ml of normal Ringer solution (145 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$) through the left ventricle, and then perfused with 200 ml of the fixative containing 2.5% glutaraldehyde and 0.5% tannic acid in 0.1 M phosphate buffer, pH 7.4. Five animals were

Accepted January 6, 1988
Received October 21, 1987

perfused with 50 ml of high K^+ Ringer solution (30 mM NaCl, 120 mM KCl, 2.3 mM $CaCl_2$) to stimulate the release of various transmitters, and then perfused with the fixative as mentioned above. The duodenum was removed and immersed in the fresh fixative for 2–3 hr. After rinsing in the phosphate buffer, the tissues were cut into small pieces and postfixed for 1 hr with 1% OsO_4 or Karnovsky's reduced OsO_4 [9] which was a mixture consisting of one part 2% OsO_4 and one part 3% potassium ferrocyanide. The tissues were dehydrated in a graded series of ethanol, passed through propylene oxide and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-700 electron microscope at 75 kV.

RESULTS

The enteric nervous system consisted of ganglia (myenteric and submucous) and unmyelinated nerve fascicles. The latter were distributed throughout the gut wall, i.e. the longitudinal and circular muscle coats, submucosa and mucosa. Most of neuronal processes in the fascicles had small clear vesicles and/or large cored vesicles which were thought to include various chemical messengers, i.e. acetylcholine, catecholamines and neuropeptides (cf. [3, 4]). Conventional synaptic junctions were sometimes found in the ganglia (Fig. 1), but rarely seen in other regions.

Omega-shaped figures indicative of exocytosis of neurotransmitters were rarely seen in the enteric nervous system of the rats perfused with normal Ringer solution. In the rats stimulated by perfusion of high K^+ Ringer solution, omega-shaped figures of transmitter-containing vesicles were frequently encountered. Therfore, the results mentioned below were obtained in the rats perfused with high K^+ Ringer solution.

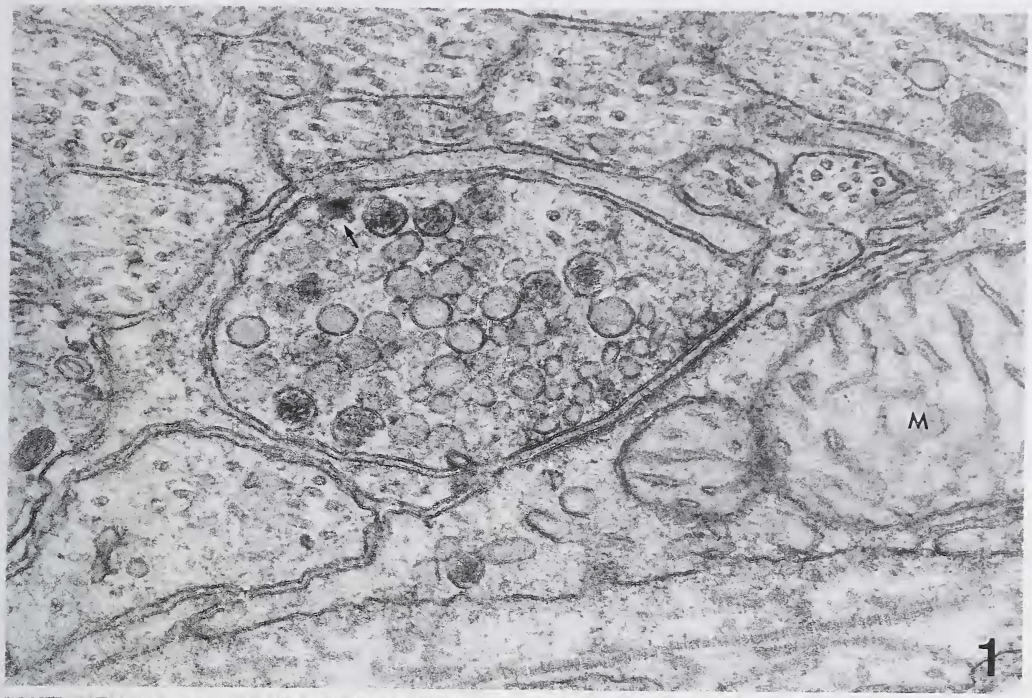
In the ganglia, small clear vesicles accumulated at the synaptic sites where characteristic thickening of cell membranes was obvious, but large cored vesicles generally located at the portion distant from the synaptic sites (Fig. 1). The exocytotic figures of large cored vesicles were found in the interspaces between neuronal processes and between neuronal process and cell body, where no membrane specialization indicative of synaptic contact was found (Fig. 1).

In the muscular coats, a large number of unmyelinated nerve fascicles were distributed among smooth muscle cells, especially in the innermost layer of the circular muscle coat (i.e. the deep muscular plexus). Unmyelinated nerve fascicles consisted of Schwann cells and many neuronal processes (Fig. 2). Irregularly-shaped processes of Schwann cells enclosed several neuronal processes. Some of neuronal processes were exposed directly to the surface of nerve fascicles. Generally, nerve fascicles and smooth muscle cells ran at a distance, and both of them were wrapped with each basement membrane (Fig. 2). A few nerve fascicles were in close contact with smooth muscle cells, where the basement membrane of both elements were absent (Fig. 3). However, the synaptic specialization was not found at these contacts (Fig. 3). Here, small clear vesicles accumulated and omega-shaped figures indicative of exocytotic release of their contents were sometimes found (Fig. 3, arrow). Omega-shaped figures of small clear vesicles also occurred in the surface of neuronal processes relatively distant from smooth muscle cells (Fig. 4, arrow). Omega-shaped figures of large cored vesicles occurred in the nerve fascicles distant from smooth muscle cells, not only in the surface of nerve fascicles (Fig. 5) but also in the interspaces between neuronal processes (Fig. 6).

In the lamina propria mucosae and the muscularis mucosae, numerous unmyelinated nerve fascicles were distributed, but the synaptic contact between neuronal processes and other cells was not found. Omega-shaped figures of large cored vesicles were found not only in the surface of nerve

Fig. 1. A synaptic ending in the myenteric ganglion. Small clear vesicles accumlate at the presynaptic site. Exocytotic figure of large cored vesicle (arrow) is seen at the site distant from the synapse. M: mitochondria. × 65,000.

Fig. 2. Unmyelinated nerve fascicle and smooth muscle cells (S) in the circular muscle coat. A nerve fascicle consists of a Schwann cell and many axons. Some axons are exposed to the surface of nerve fascicle. F: fibroblasts, N: nucleus of Schwann cell. ×16,000.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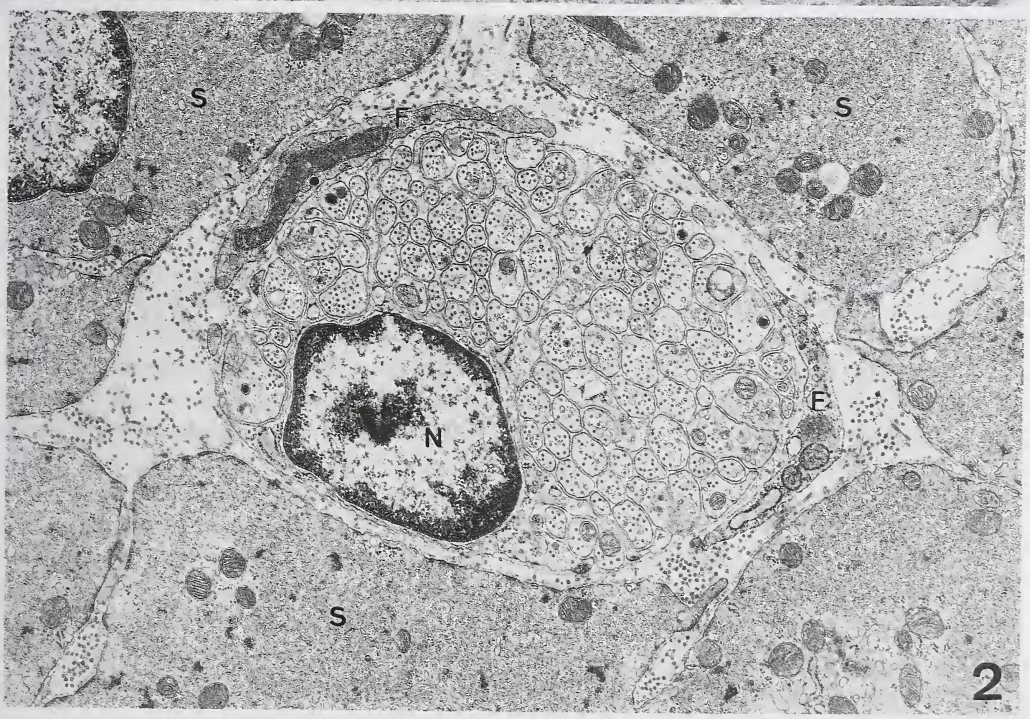
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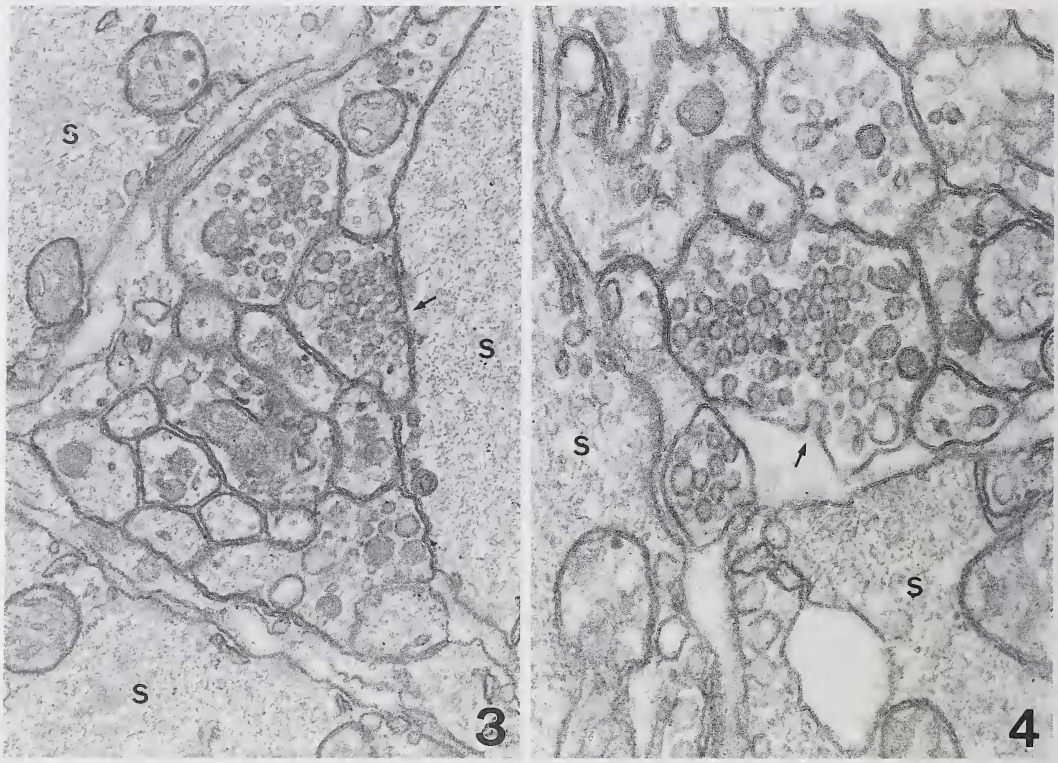

FIG. 3. A nerve fascicle in close contact with smooth muscle cell (S) in the circular muscle coat. Exocytotic figure of small clear vesicle (arrow) is seen. ×40,000.

FIG. 4. Exocytotic figure of small clear vesicle (arrow) at the site distant from smooth muscle cell (S) in the circular muscle coat. ×55,000.

fascicles (Fig. 7) but also in the interspaces between neuronal processes and between neuronal and Schwann cell's processes (Fig. 8).

In spite of applying the tannic acid fixation, the electron density of the exocytosed vesicles was relatively low when compared with the results of previous studies [1, 2, 7, 8]. This may be due to the use of Karnovsky's reduced OsO_4 [9] which is known as the fixative preserving membrane structure excellently.

DISCUSSION

In the present study, I demonstrated by electron microscopy that the exocytosis of large cored vesicles and small clear vesicles occurred in the terminal varicosities of the enteric neurons. The exocytosis theory has been commonly accepted as a release mechanism of secretory granules from the neurosecretory cells, various endocrine and exocrine cells (cf. [6, 10, 11]). However, there have been only a few studies which demonstrated the exocytotic release of neurotransmitters in the authentic neurons. According to Nagasawa [6], the frequency of encountering the exocytotic figures is extremely rare in the neurosecretory axon terminals of the posterior pituitary when compared with other secretory cells such as the anterior pituitary or the adrenal medulla. But the stimulation of a solution containing high KCl and $CaCl_2$ increases the frequency of the exocytotic figures. In the present study, similar phenomenon was found also in the enteric neurons. Although the detailed mechanism of KCl-induced exocytosis is not fully understood, the sites and mode of transmitter release seem to be similar to those of the normal condition.

Generally, the exocytotic release of small clear